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(54) Mutant luciferase of a firefly, mutant luciferase genes, novel recombinant DNAS containing the genes and a method of producing mutant luciferase

Mutierte Luciferase vom Leuchtkäfer, mutiertes Luciferasegen, rekombinante DNS diese Gene enthaltend, und Verfahren zur Herstellung von mutierter Luciferase

Une luciférase mutante de luciole, gènes de luciférase mutante, nouvel ADN recombinant contenant ces gènes et méthode de production de luciférase mutante

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02.10.1991 Bulletin 1991/40

(73) Proprietor: **KIKKOMAN CORPORATION**
Noda-shi, Chiba-ken (JP)

(72) Inventors:
- **Kajiyama, Naoki**
  Noda-shi, Chiba-ken (JP)
- **The other inventor has agreed to waive his entitlement to designation.**

(74) Representative: **Cresswell, Thomas Anthony et al**
J.A. KEMP & CO.
14 South Square
Gray's Inn
London WC1R 5LX (GB)

(56) References cited:
EP-A- 0 004 913
EP-A- 0 301 541

- **PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 82, no. 23, December 1985, WASHINGTON US pages 7870 - 7873; J.R. de Wet et al.**
- **SCIENCE. vol. 244, no. 4905, 12 May 1989, LANCASTER, PA US pages 700 - 702; K. V. Wood et al.: "Complementary DNA coding click beetle luciferases can elicit bioluminescence of different colors"**

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The nucleotide sequence of a mutant luciferase gene can be determined according to the method as described in Section 17 of Example.

The transformants capable of producing mutant luciferase may be cultured in a solid medium, but a liquid culture medium is preferable.

Suitable medium includes more than one nitrogen source among yeast extract, tryptone, peptone, meat extract, corn steep liquor and exudate of soybean or wheat, and more than one inorganic salt among NaCl, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, magnesium sulfate, magnesium chloride, ferric chloride, ferric sulfate, manganese sulfate and the like, and if necessary, some carbohydrates and vitamins.

The pH of the culture medium is preferably adjusted to 7-9. Incubation may be carried out at 30-42°C for 4-24 hours, preferably 37°C for 6-8 hours in a submerged aeration culture, a shaking culture, or a stationary culture.

After incubation, mutant luciferase is recovered from the culture according to the methods known in the art: Mutant luciferase is recovered by disrupting cells using sonication, mechanical and enzymatic (e.g., lysozyme) lysis or by incubating cells in the presence of toluene with or without shaking and allowing cells to secrete enzyme into the medium. The lysate is filtered or centrifuged to remove cells and cell debris. If it is necessary to remove nucleic acid, streptomycin sulfate, protamine sulfate or manganese sulfate was added to the filtrate or the supernatant. The mixture is then fractionated using ammonium sulfate, alcohol or acetone. The precipitate recovered contains crude luciferase.

Crude enzyme thus obtained may be purified by a method or the combination of methods which includes a gel filtration method using Sephadex, Ultro-Gel or Bio-Gel, adsorption chromatography using an ion-exchanger or hydroxyapatite, affinity chromatography, polyacrylamide gel electrophoresis, sucrose density gradient centrifugation, and fractional filtration using a molecular sieve and hollow fiber membrane.

Purified luciferase is characterized as follows:

Mutant luciferase catalyzes luciferin to produce colours of light, orange (wavelength: 595 nm and 607 nm), red (609 nm and 612 nm) and green (558 nm). The other physical and chemical properties of mutant luciferase of Luciora cruciata are found identical to those of native luciferase as described in the Japanese Patent Appln. LOP Publication No. 141592/1989.

The Effect of the Invention

The present invention provides industrially useful luciferase. Mutant luciferase of the invention is produced by culturing a microorganism belonging to the genus Escherichia which carries the recombinant DNA containing the mutant luciferase gene of a firefly. Mutant luciferase can produce red, orange and green colours of light which are not seen by native luciferase. Mutant luciferase can be used to measure ATP accurately in a coloured solution such as red (e.g., blood), orange, or green colours in which native luciferase has not provided reliable results.

Description of the Figures

Fig. 1 shows the restriction map of a recombinant plasmid pALf3.

Fig. 2 shows the restriction map of a recombinant plasmid pGLf1.

Example

The following example further illustrates the invention.

The following Sections 1-10 describe the construction of a recombinant DNA containing the luciferase cDNA of Photinus pyralis (Photinus pyralis is a member of fireflies). The construct is used as a probe to screen the luciferase gene of Luciora cruciata.

(1) Preparation of a Luciferase mRNA of Photinus pyralis

1 g of dried tails of Photinus pyralis (Sigma) was ground well using a mortar and a pestle. 5 ml of buffer [20 mM Tris-HCl/pH 7.4, 10 mM NaCl, 3 mM magnesium acetate, 5% (w/v) sucrose, 1.2% (v/v) Triton X-100, 10 mM vanadyl nucleoside complex (New England Biolabs)] was added to the material. The material was further ground as described above.

5 ml of the solution thus obtained was placed in a blender cup (Nippon Seiki Seisakusho Co.) and mixed at 5,000 r.p.m. for 5 minutes. 12 ml of a guanidine isothiocyanate solution [6 M guanidine isothiocyanate, 37.5 mM sodium citrate/pH 7.0, 0.75% (w/v) sodium N-lauroylsarcosinate, 0.15 M β-mercaptoethanol] was added to the mixture. The mixture was mixed in a blender cup at 3,000 r.p.m. for 10 minutes. Then, the mixture was filtrated through a three-folded gauze. The filtrate was layered onto four ultra-centrifuge tubes (Hitachi Koki Co.) containing 1.2 ml each of 5.7 M cesium chloride. The tube was ultracentrifuged (SCP55H, Hitachi Koki Co.) at 30,000 for 16 hours at 15°C. The

Gene 25: 263, 1983, as recommended by the manufacturer's instructions.

150 ng of cDNA was suspended in 7 μℓ of TE (10 mM Tris-HCl/pH 7.5, 1 mM EDTA). 11 μℓ of buffer (280 mM sodium cacodylate/pH 6.8, 60 mM Tris-HCl/pH 6.8, 2 mM cobalt chloride), 3.8 μℓ of a tailing solution [7.5 μℓ of 10 mM dithiothreitol, 1 μℓ of poly A (10 ng/ml), 2 μℓ of 5 mM dCTP, 110 μℓ of water] and 29 units of terminal transferase (Boehringer Mannheim Inc.) were added to the suspension. The mixture was incubated at 30°C for 10 minutes. After incubation, 2.4 μℓ of 0.25 M EDTA and 2.4 μℓ of 10% (w/v) sodium dodecylsulfate were added to the mixture to stop the reaction.

25 μℓ of phenol equilibrated with water was added to the mixture. The aqueous phase was saved. 25 μℓ of 4 M ammonium acetate and 100 μℓ of ice cold ethanol were added to the aqueous portion. The mixture was incubated at -70°C for 15 minutes. After incubation, the mixture was centrifuged at 12,000 r.p.m. for 10 minutes. The pellet was removed and resuspended in 10 μℓ of TE. The resulting suspension contained 100 ng of deoxycytidine-tailed cDNA.

(5) Preparation of a Vector pMCEI0

pKN305 and pMCI403-3 (Japanese Patent Appln. LOP Publication No. 274683/1986) were constructed using E. coli W3110 (ATCC 27325), pBR325 (BRL), pBR322 (Takara Shuzo Co., LTD) according to the method described by Masuda, T. et. al., (1986, Agricultural Biological Chemistry 50: 271-279). 1 μg of pKN305 DNA and 1 μg of pMCI403-3 DNA were dissolved in 10 μℓ of a solution (50 mM Tris-HCl/pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol) in a separate tube. 2 units of HindIII and 2 units of SalI (Takara Shuzo Co., LTD) were added to each tube. The mixture was incubated at 37°C for an hour. After digestion, the mixture was extracted with phenol. The extract was then precipitated with ethanol. The precipitate was dissolved in 10 μℓ of ligation buffer (20 mM $MgCl_2$, 60 mM Tris-HCl/pH 7.6, 1 mM ATP, 15 mM dithiothreitol). 1 unit of T4 DNA ligase (Takara Shuzo Co., LTD) was added to the solution and the mixture was incubated at 20°C for 4 hours. The mixture was used to transform JM101 (ATCC 33876) according to the method (J. Bacteriology, 1974, 119: 1072-1074). The transformants were screened on an agar plate containing ampicillin and tetracycline in addition to a necessary culture medium. The transformants were then further screened for the β-galactosidase activity. After screening, a positive colony was found and designated JM101 (pMCE10). The recombinant plasmid contained was designated pMCE10. JM101 (pMCE10) was cultured at 37°C for 16-24 hours. 20 ml of the culture was added to 1 ℓ of a culture medium [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl]. The mixture was incubated at 37°C for three hours. At three hours of incubation, 0.2 g of chloramphenicol was added to the mixture. The mixture was further incubated at 37°C for 20 hours.

The culture was centrifuged at 6,000 r.p.m. for 10 minutes to give 2 g of the cells which were suspended in 20 ml of 350 mM Tris-HCl/pH 8.0 buffer containing 25% (w/v) sucrose. 10 mg of lysozyme, 8 ml of 0.25 M EDTA/pH 8.0 and 8 ml of 20% (w/v) sodium dodecylsulfate were added to the suspension. The mixture was incubated at 60°C for 30 minutes.

13 ml of 5 M NaCl was then added to the mixture. The mixture was further incubated at 4°C for 16 hours. After incubation, the mixture was centrifuged at 15,000 r.p.m. for 30 minutes. The supernatant was extracted with phenol. Then, DNA was precipitated with ethanol.

The precipitate was dried under reduced pressure and then dissolved in 6 ml of TE. 6 g of cesium chloride and 0.2 ml (10 mg/ml) of ethidium bromide were added to the solution. The mixture was ultracentrifuged at 39,000 r.p.m. for 42 hours. After centrifugation, pMCEI0 DNA portion was removed and extracted with n-butanol to remove ethidium bromide. The DNA solution was then dialyzed against TE. After dialysis, the dialysate contained 500 μg of DNA.

(6) Preparation of Deoxyguanidine-tailed Vector pMCEI0 and pUCI9

15 μg of pMCEI0 was dissolved in 90 μℓ of TE. 10 μℓ of Med buffer [100 mM Tris-HCl/pH 7.5, 100 mM $MgCl_2$, 10 mM dithiothreitol, 500 mM NaCl] and 30 units of AccI (Takara Shuzo Co., LTD) were added to the solution. The mixture was incubated at 37°C for an hour. After digestion, the mixture was extracted with 100 μℓ of phenol equilibrated with water. To the extract, 1/10 volume of 3 M sodium acetate/pH 7.5 and two volumes of ice cold ethanol were added. The mixture was incubated at -70°C for 15 minutes. After incubation, the mixture was centrifuged at 12,000 r.p.m. for 10 minutes. The pellet was resuspended in 10 μℓ of TE.

15 μℓ of a solution (280 mM sodium cacodylate/pH 6.8, 60 mM Tris-HCl/pH 6.8, 2 mM cobalt chloride), 5 μℓ of a tailing solution [7.5 μl of 10 mM dithiothreitol, 1 μℓ of poly A (10 ng/ml), 2 μℓ of 5 mM dGTP, 110 μℓ of water] and 5 units of terminal transferase (Takara Shuzo Co., LTD) were added to the mixture. The mixture was incubated at 37°C for 15 minutes. The rest of the procedure was carried out as described in Section 4. The resulting solution contained DNA of pMCEI0 with a deoxyguanosine tail at the AccI site.

DNA of pUCI9 with a deoxyguanosine tail at the PstI site was prepared as follows: 30 μg of pUCI9 (Takara Shuzo Co., LTD) was dissolved in 350 μℓ of TE. 40 μℓ of Med buffer and 120 units of PstI (Takara Shuzo Co., LTD) were added to the solution. The mixture was incubated at 37°C for an hour. After digestion, the mixture was extracted with

restriction fragments were electrophoresed on a 5% (w/v) polyacrylamide gel according to the method described by Maxam, A. (1980, Methods in Enzymology 65: 506). 1 μg of the 190 bp Sau3AI fragment containing the luciferase cDNA was isolated as described above.

1 μg of the fragment was labelled with α-^{32}P-dCTP (Amersham) using a kit (Takara Shuzo Co., LTD) according to the nick translation method (J. Mol. Biol., 1977, 113: 237-251 and Molecular Cloning, 1982, Cold Spring Harbor Laboratory, NY, pp 109-112).

(10) Screening of the Luciferase cDNA Library in the pUC19 Vector Using the ^{32}p-Labelled Probe

The luciferase cDNA library in the pDC19 vector was screened using the ^{32}p-labelled probe according to the colony hybridization method (Proteins, Nucleic Acid, Enzyme, 1981, 26: 575-579). Positive colonies were obtained and one of colonies was designated as pALf3. pALf3 DNA was prepared as described in Section 5 and used to transform E. coli DHI. The transformant was designated as DHI (pALf3). DHI (pALf3) was deposited as ATCC 67462.

pALf3 DNA was digested with one or two enzymes from the group consisting of XbaI, HindIII, BamHI, EcoRI and PstI (Takara Shuzo Co., LTD). For a molecular weight marker, λDNA (Takara Shuzo Co., LTD) was digested with HindIII. The restriction fragments were electrophoresed on an agarose gel. The band patterns of digested pALf3 DNA were compared with those of the λDNA marker. The size of the luciferase cDNA fragment was found to be 1,700 bp. The restriction map of pALf3 is shown in Fig. 1.

(11) Preparation of Luciferase mRNA of Luciola cruciata

10 g of living Luciola cruciata (purchased from Seibu Department Store) was placed in an ultracold freezer. After the insect was frozen they were taken out from the freezer. The tails of fireflies were cut off with scissors to yield 2 g of the tails. 18 ml of a guanidine isothiocyanate solution was added to 2 g of the tails. 1.1 mg of total RNA was recovered and loaded onto the top of an oligo (dT) cellulose column according to the method as described in Section 1. 30 μg of luciferase mRNA was recovered.

(12) Construction of the Luciferase cDNA Library of Luciola cruciata

cDNA was prepared using a kit (Amersham) according to the method (Mol. Cell. Biol., 1982, 2: 161 and Gene, 1983, 25: 263).

0.9 μg of double-stranded cDNA was prepared from 2 μg of mRNA. 0.3 μg of cDNA was polydeoxycytidine-tailed according to the method as described in Section 4.

20 ng of polydeoxycytidine-tailed cDNA and 500 ng of polydeoxyguanocine-tailed pUC19 DNA (see Section 6) were annealed according to the method as described in Section 7. The construct was used to transform E. coli DHI (ATCC 33849) according to the method described by Hanahan (1985, DNA Cloning, 1: 109-135).

(13) Screening of the Luciferase cDNA Library

10 μg of pALf3 DNA as described in Section 10 was dissolved in 90 μℓ of TE. 10 μℓ of Med buffer, 25 units each of EcoRI and ClaI (Takara Shuzo Co., LTD) were added to the solution. The mixture was incubated at 37°C for 2 hours. After digestion, the restriction fragments were electrophoresed on an agarose gel. The 800 bp EcoRI-ClaI fragment containing luciferase cDNA was isolated. 1 μg of the DNA fragment recovered was labelled with α-^{32}P-dCTP (Amersham) according to the nick translation method. The luciferase cDNA library of Luciora cruciata was screened using the ^{32}P-labelled probe according to the colony hybridization method. Several positive colonies were obtained and one of the colonies was designated at pGLf1. pGLf1 DNA was prepared according to the method as described in Section 5, and used to transform E. coli DHI. The transformant was designated as DH1 (pGLf1). DHI (pGLf1) was deposited as ATCC 67482.

pGLf1 DNA was digested with one or two enzymes from the group consisting of HpaI, HindIII, EcoRV, DraI, AflII, HincII, PstI (Takara Shuzo Co., LTD) and SspI (New England Bio-Laboratory). For a molecular weight marker, λ phage DNA (Takara Shuzo Co., LTD) was digested with HindIII. The restriction fragments were electrophoresed on an agarose gel. The band patterns were analyzed. The fragment containing luciferase cDNA was 2,000 bp. The restriction map of pGLf1 is shown in Fig. 2.

(14) DNA Sequencing of the Luciferase cDNA of Luciola cruciata

10 μg of pGLf1 DNA was digested with PstI (Takara Shuzo Co., LTD). After digestion, 2.5 μg of the 2.0 kb DNA fragment containing the luciferase cDNA was recovered. The 2.0 kb fragment was inserted into the PstI site of pUC119

1 μg of the 4.0 kb fragment, 1 μg of the 500 bp fragment and 0.1 μg each of the oligomer phosphorylated as above were dissolved in 8 μℓ of water. 1 μℓ of 10x ligation buffer (200 mM $MgCl_2$, 660 mM Tris-HCl/pH 7.6, 10 mM ATP, 150 mM dithiothreitol) and 1 μℓ (1 unit) of T4 DNA ligase (Takara Shuzo Co., LTD) were added to the mixture. The mixture was incubated at 16°C for 16 hours. Then, the mixture was used for transformation. Transformation of JM101 (ATCC 33876) and isolation of plasmid DNA were carried out in an analogous way as in Section 5. Plasmid DNA was digested with one or two enzymes from the group consisting of SspI, EcoRV and PstI. The restriction fragments were electrophoresed on a 0.7% agarose gel. The recombinant plasmid containing the trp promoter and the luciferase cDNA was selected and designated as pGLf37. A JM101 transformant carrying pGLf37 was designated as JM101 (pGLf37).

(16) Mutagenesis of Recombinant Plasmid pGLf37

30 μg of pGLf37 DNA was dissolved in 100 μℓ of a hydroxylamine solution (0.8 M hydroxylamine hydrochloride, 0.1 M phosphate buffer/pH 6.8, 1 mM EDTA). The mixture was incubated at 65°C for 2 hours. After incubation, DNA was precipitated with ethanol in conventional way. The precipitate was resuspended in TE (10 mM Tris-HCl/pH 7.5, 1 mM EDTA). The mixture was used to transform E. coli JM101 (ATCC 33876) according to the method described by Hanahan (1985, DNA Cloning, 1: 109-135). The mixture containing the transformants was plated out on an LB-amp agar plate [1% (w/v) bactotrypton, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 50 μg/ml of ampicillin, 1.4% (w/v) agar]. The plate was incubated at 37°C for 12 hours. A colony appeared on the plate was inoculated into 3 ml of an LB-amp medium [1% (w/v) bactotrypton, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 50 μg/ml of ampicillin] and incubated with shaking at 37°C for 18 hours. 0.5 ml of the culture was added to 10 ml of an LB-amp medium. The mixture was incubated with shaking at 37°C for 4 hours. After incubation, the mixture was centrifuged at 8,000 r.p.m. for 10 minutes.

20 mg of the cells collected was suspended in 0.9 ml of a buffer (0.1 M KH_2PO_4/pH 7.8, 2 mM EDTA, 1 mM dithiothreitol, 0.2 mg/ml of protamine sulfate). 100 μℓ of lysozyme solution (10 mg/ml) was added to the suspension. The mixture was placed on ice for 15 minutes. The mixture was then frozen in a dry ice/methanol bath. The mixture was removed from the bath and left standing at 25°C. When the mixture was completely thawed, it was centrifuged at 12,000 r.p.m. for 5 minutes. 1 ml of a supernatant containing crude enzyme was obtained.

50 μℓ of the crude enzyme solution thus obtained was added to 400 μℓ of a luciferin/ATP mixture [260 μℓ of 25 mM glycylglycine/pH 7.8, 16 μℓ of 0.1 M magnesium sulfate, 24 μℓ of 1 mM luciferin (Sigma), 100 μℓ of 10 mM ATP] to observe the colour of the light. There were six types of colours: red (609 nm and 612 nm), orange (595, 607), green (two 558's).

Alternatively, crude enzyme was purified according to the method described in the Japanese Patent Appln. LOP Publication No. 141592/1989, tested, and found the same colour displayed as described above.

Recombinant DNAs encoding mutant luciferase which produces red colours of light (609 nm and 612 nm) were designated as pGLf37C-M-2 and pGLf37C-M-5, respectively. E. coli JM101 was transformed with pGLf37C-M-2 or pGLf37C-M-5. The transformants, E. coli JM101 (pGLf37C-M-2) and JM101 (pGLf37C-M-5) were deposited with Fermentation Research Institute, Agency of Industrial Science and Technology and were assigned the accession number FERM BP-2825 and FERM BP-3136, respectively. Recombinant DNAs encoding mutant luciferase which produces orange colours of light (595 nm and 607 nm) were designated as pGLf37C-M-4 and pGLf37C-M-1, respectively. The transformants, E. coli JM101 (pGLf37C-M-4) and JM101 (pGLf37C-M-1), were deposited with the same and were assigned the accession number FERM BP-2826 and FERM BP-3135, respectively. Recombinant DNAs encoding mutant luciferase which produces green colours of light (two wavelengths of 558 nm) were designated as pGLf37C-M-6 and pGLf37C-M-7, respectively. The transformants, E. coli JM101 (pGLf37C-M-6) or JM101 (pGLf37C-M-7), were deposited with the same and were assigned the accession number FERM BP-3137 and FERM BP-3138, respectively.

Table I summarizes the colour of light, the position of mutation in the nucleotide sequence and the position of mutation in the amino acid sequence of the bacterial strains.

(ii) TITLE OF INVENTION:
(iii) NUMBER OF SEQUENCES:
(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE:
(B) STREET:
(C) CITY:
(D) STATE:
(E) COUNTRY:
(F) ZIP:

(v) COMPUTER READABLE FORM;

(A) MEDIUM TYPE:
(B) COMPUTER:
(C) OPERATING SYSTEM:
(D) SOFTWARE:

(vii) ATTORNEY/AGENT INFORMATION:

(A) NAME:
(B) REGISTRATION NUMBER:
(C) REFERENCE/DOCKET NUMBER:

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE:
(B) TELEFAX:

(2) INFORMATION FOR SEQ ID NO: 1

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1644 Base pairs
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA
(vi) ORIGINAL SOURCE
(A) ORGANISM: Luciola cruciata
(ix) FEATURES
(A) OTHER INFORMATION
Luciferase cDNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1

```
          850         860         870         880         890         900
GATTATAAAT GTACAAGTGT TATTCTTGTA CCGACCTTGT TTGCAATTCT CAACAAAAGT

          910         920         930         940         950         960
GAATTACTCA ATAAATACGA TTTGTCAAAT TTAGTTGAGA TTGCATCTGG CGGAGCACCT

          970         980         990        1000        1010        1020
TTATCAAAAG AAGTTGGTGA AGCTGTTGCT AGACGCTTTA ATCTTCCCGG TGTTCGTCAA

         1030        1040        1050        1060        1070        1080
GGTTATGGTT TAACAGAAAC AACATCTGCC ATTATTATTA CACCAGAAGG AGACGATAAA

         1090        1100        1110        1120        1130        1140
CCAGGAGCTT CTGGAAAAGT CGTGCCGTTG TTTAAAGCAA AAGTTATTGA TCTTGATACC

         1150        1160        1170        1180        1190        1200
AAAAAATCTT TAGGTCCTAA CAGACGTGGA GAAGTTTGTG TTAAAGGACC TATGCTTATG

         1210        1220        1230        1240        1250        1260
AAAGGTTATG TAAATAATCC AGAAGCAACA AAAGAACTTA TTGACGAAGA AGGTTGGCTG

         1270        1280        1290        1300        1310        1320
CACACCGGAG ATATTGGATA TTATGATGAA GAAAAACATT TCTTTATTGT CGATCGTTTG

         1330        1340        1350        1360        1370        1380
AAGTCTTTAA TCAAATACAA AGGATACCAA GTACCACCTG CCGAATTAGA ATCCGTTCTT

         1390        1400        1410        1420        1430        1440
TTGCAACATC CATCTATCTT TGATGCTGGT GTTGCCGGCG TTCCTGATCC TGTAGCTGGC

         1450        1460        1470        1480        1490        1500
GAGCTTCCAG GAGCCGTTGT TGTACTGGAA AGCGGAAAAA ATATGACCGA AAAAGAAGTA

         1510        1520        1530        1540        1550        1560
ATGGATTATG TTGCAAGTCA AGTTTCAAAT GCAAAACGTT TACGTGGTGG TGTTCGTTTT

         1570        1580        1590        1600        1610        1620
GTGGATGAAG TACCTAAAGG TCTTACTGGA AAAATTGACG GCAGAGCAAT TAGAGAAATC

         1630        1640
CTTAAGAAAC CAGTTGCTAA GATG
```

(3) INFORMATION FOR SEQ ID NO: 2

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 548 Amino acids
(B) TYPE: Amino acid
(C) STRANDEDNESS: -
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide
(vi) ORIGINAL SOURCE
(A) ORGANISM: Luciola cruciata
(ix) FEATURES
(A) OTHER INFORMATION
Luciferase
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2

(C) STRANDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleric acid Oligomer
(ix) FEATURES
(A) OTHER INFORMATION
A part of SD-ATG DNA fragment of trp promoter +27 nucleotides of N-terminal of wild type luciferase
(xi) SEQUENCE DESCRIPTION: SEQ ID:NO 3

CGACAATGGAAAACATGGAAAACGATGAAAAT

(5) INFORMATION FOR SEQ ID NO:4

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 bases
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid Oligomer
(ix) FEATURES
(A) OTHER INFORMATION
A part of SD-ATG DNA fragment of trp promoter +27 nucleotides of N-terminal of wild type luciferase
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4

ATTTTCATCGTTTTCCATGTTTTCCATTGT

Claims

1. A mutant luciferase which has the sequence of Seq. ID No. 2, altered to contain at least one amino acid substitution selected from: 233 Val to Ile; 239 Val to Ile; 286 Ser to Asn; 326 Gly to Ser; 433 His to Tyr; and 452 Pro to Ser.

2. A mutant luciferase gene encoding the amino acid sequence of a mutant luciferase as claimed in claim 1.

3. A recombinant DNA comprising a mutant luciferase gene as claimed in claim 2.

4. A recombinant DNA according to claim 3 which has the sequence of Seq. ID No. 1, altered to contain at least one substitution resulting in a coding change which provides the mutant luciferase of claim 1.

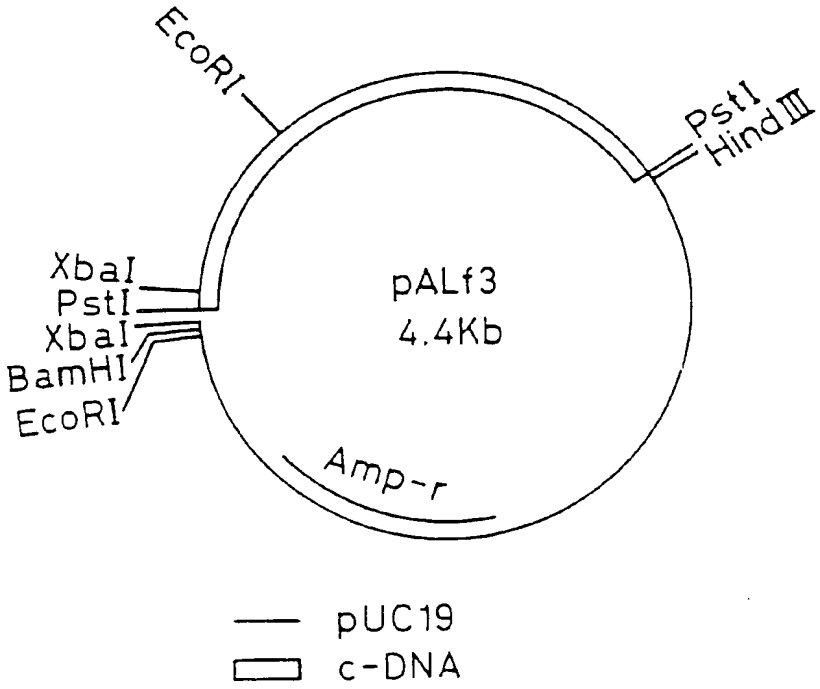
5. A method of producing a mutant luciferase which comprises culturing in a medium a microorganism belonging to the genus *Escherichia* transformed with a recombinant DNA as defined in claim 3 or 4 and recovering the mutant luciferase from the culture.

6. Use of a mutant luciferase as claimed in claim 1 for measuring the amount of ATP in a coloured solution.

7. An ATP assay kit comprising a mutant luciferase as claimed in claim 1 and luciferin.

8. A microorganism belonging to the genus *Escherichia* transformed with a recombinant DNA as claimed in claim 3 and selected from FERM BP-2825, FERM BP-2826, FERM BP-3135, FERM BP-3136, FERM BP-3137 and FERM BP-3138.

FIG. 1


EcoRI
PstI
HindIII
XbaI
PstI
XbaI
BamHI
EcoRI
pALf3
4.4Kb
Amp-r
pUC19
c-DNA